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Detection of Beta-Lactamase Variants in Municipal Wastewater and Fresh Water

Sunil Pandey
Eastern Illinois University

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Detection of Beta-Lactamase Variants in Municipal Wastewater and Fresh Water

BY

Sunil Pandey

THESIS

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Abstract

The occurrence and spread of antibiotic-resistant genes (ARGs) are pressing public health problems worldwide. A key factor contributing to the spread of ARGs is lateral gene transfer. Wastewater treatment plants (WWTPs) are measured hot spots of microbial diversity and resistance because they receive polluted wastewater from diverse sources and contain a variety of different environments with dense bacterial loads. Due to the overuse of antibiotics the genetic capacities of microbes have profited. This helps every source of resistance gene and every means of horizontal gene transmission to develop the multiple mechanism of resistance to each antibiotic used clinically, agriculturally, or by any other medium. In wastewater treatment plants, where gastrointestinal wastes from city residents co-mingle, the probability for lateral gene transfer events is greatly increased. In this study, we use PCR technique to detect four beta-lactamase loci to assess the prevalence of ARGs. Wastewater samples from municipal plant at different stages of treatment as well as water samples from the river upstream and downstream from the release site were collected, followed by total DNA extraction and purification. These were then used as templates in PCR-based detection of beta-lactamase (bla) resistance loci. Our results showed the presence of four loci (blaKPC, blaTEM, blaSHV,blaAMPC) in influx, secondary treatment wastewater but not in the efflux, nor in the river water samples. Up to now we can say there is no detectable levels of ARGs in WWTP effluent samples, upstream and the downstream rivers. These data are vital in understanding the role of WWTPs in contributing to the spread of antibiotic resistance in the environment.

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List of Abbreviations

ARB- Antibiotic resistant bacteria

ARG- Antibiotic resistant gene

EPA: United States Environmental Protection Agency

GlcNAc, NAM: N-acetyl glucosamine

MurNA, NAM: N-acetyl muramic acid

GT: Glycosyltransferase

TP: Transpeptidase

PBP: Penicillin binding protein

Bla: Beta-Lactamase

OMP: Outer Membrane protein

ESBL: Extended spectrum beta lactamases

IRT: Inhibitor-resistant TEM

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

WWTP: Wastewater treatment plant

PCR: Polymerase chain reaction

TBE: Tris-borate-EDTA

EDTA: Ethylenediaminetetraacetic acid

TAE:Tris- acetate-EDTA

1. Introduction

Over the past several decades, antibiotic-resistant bacterial infections have become gradually more prevalent, increasing morbidity and mortality as well as the cost of treatment to the patients [1-2]. The incidence and spread of antibiotic-resistant bacteria (ARB) are persistent public health problems globally, and aquatic ecologies are a recognized reservoir for antibiotic resistant bacteria and antibiotic resistance genes (ARGs) [3]. Naturally occurring ARGs and antibiotic-resistant bacteria in the water are selected for and supplemented for by antibiotics found in sewage and agricultural environments, which is a result of the increase in the use of antibiotics [3]. The increasing interest in the international community and many other research organizations in ARGs persisting in the environment is due to the high prevalence and incidence of the ARGs report published by many research organizations and individuals. Spread of the antibiotic resistant gene in the environment can be studied in different ways including the culture and molecular methods. When the wastewater from individual homes, agricultural farms, hospitals and industrial facilities carrying different microbial human gastrointestinal flora drains directly to the wastewater treatment plant, there will be a high load of microbial flora in the mixture. These microbial populations contain different variants carrying the antibiotic resistant genetic markers, thus enabling lateral gene transfer that helps to spread the antibiotic resistant. Untreated water from the wastewater, local stream, ponds, rivers are the common places that may have combination of different microorganisms' load which can be useful to identify the resistant gene in vitro [4]. Advanced treatment technologies in the wastewater and surface water

and different disinfection process are regarded as main tool to control the spread of antibiotic resistant gene into the environment. Despite these hard efforts made over the last years to bring solutions to control antibiotic resistance spread in the environment, there are still vital gaps to fill in. Investigating the prevalence and incidence of antibiotic resistant loci in the environmental sample is one of the main steps in determining and monitoring the spreading of ARGs via wastewater [4-5]. Sewage from hospital, followed by municipal, agricultural, and aquatic wastewater are shown to be important sources of these many resistant genes and resistant bacteria in the environment [6].

1.1. Wastewater treatment plant and process.

Wastewater is used water which can also an important resource of water, particularly with periodic droughts and water shortages in many areas of the world. However, wastewater contains many harmful substances that directly or indirectly impact humans and animals around the globe. Therefore, wastewater cannot be released back into the environment until it is treated. Treating wastewater fulfills two important purposes: to restore the water supply and to protect the planet from toxins and harmful microorganisms. In the process, microbial organisms are indispensable, because they consume the useable carbon, nitrogen, and other nutrients from the wastewater. The varying metabolic capacities from different microbes are employed at different stages of the process to achieve the final objective of purifying wastewater.

The basic purpose of wastewater treatment is to speed up the natural procedures by which water is purified through variety of methods. Once, it is collected through the local pipes collected the municipal treatment of wastes begins. Wastewater

travels through the pipes by gravity and sometimes pipes get too deep, which require a pumper lift station to move wastewater to a new section of pipes depending on gravity again [7]. In the primary stage, solids can settle and be removed from wastewater. In the secondary stage, biological courses are used to further purify wastewater [8]. According to the United States Environmental Protection Agency (US EPA), there are about 800,000 miles of collection systems along with 500,000 miles of private laterals which connect properties to wastewater treatment plants. Treatment facilities in the United States process about 34 billion gallons of wastewater every day [9].

Typically, following the primary treatment which eliminates the large floating objects such as rags and sticks that might clog pipes or damage equipment, wastewater is then transferred into a grit chamber, where cinders, sand, and small stones settle to the bottom. After screening is finalized and grit has been removed, sewage will encompass organic and inorganic matter laterally with other suspended solids. These solids are very tiny particles that can be removed from sewage in a sedimentation tank. When the speed of the flow through one of these tanks is lowered, the suspended solids will initially sink to the bottom, where they form a mass of solids called raw primary biosolids or sludge. Biosolids are usually removed from tanks and even these can be used as a fertilizer [10-11].

The secondary phase of treatment removes about 85 percent of the organic matter in sewage by employing microbes. The main secondary treatment methods used in secondary treatment are the dripping filter and the activated sludge process. After effluent passes from the sedimentation tank in the primary stage it flows or is

pumped to a facility using one or the other of these processes. After secondary treatment where activated sludge is digested by the microbes to remove dissolved carbons, the wastewater is further treated to remove dissolved nitrogen and sulfur, as needed. Following that, part of the sample may be sent to anaerobic digester for further processing. Samples are then allowed to sediment and clarify. At this point, chlorination or UV treatment may be employed, prior to discharging the water back into the environment.

A successful treatment of wastewater will achieve two goals: To remove potential pathogens, and to remove dissolved organic molecules so we do not cause eutrophication of the aquatic systems.

1.2. Antibiotic resistance beta-lactam

The modern age of antibiotics began with the discovery of penicillin, which is a member of the class of antibiotic compounds known as beta-lactams by Sir Alexander Fleming in 1928[14-15]. Subsequently, antibiotics have improved modern medicine and saved millions of lives [figure 1] [16-17]. Since then, new beta-lactam antibiotics were discovered, developed, and deployed[18-19]. In the recent years, beta-lactam antibiotic has become the greatest commonly used antibiotic. Beta-lactams is the most prescribed for injectable antibiotics(65%) in the United States[Figure 2] [20]. The most common forms of beta-lactam drugs are penicillin's, cephalosporins, and monobactams.

These drugs are prescribed to cure illness caused by bacterial infections, such as diarrhea, hypersensitivity, nausea, rash, neurotoxicity and urticaria. Despite the

benefits, the day-by-day increases in consumption of antibiotics has now rendered the resistance to antibiotics one of the major global public health threats. The very first antibiotic penicillin resistance became a considerable clinical problem by the 1950s and other infections [18-21]. Unfortunately, resistance has eventually been seen to nearly all antibiotics that have been developed (Figure 2)[23].

The structure of most bacteria consists of cell membrane surrounded by cell wall. The bacterial cell wall is a flexible macromolecule protecting the bacterium, enabling it to resist lysis caused by high intracellular osmotic pressure. Bacterial cell walls are made of glycan strands linked together to obtain a polymer called peptidoglycan. Peptidoglycan consists of two alternating saccharides, N-acetyl glucosamine (GlcNAc, NAG) and N-acetyl muramic acid (MurNA, NAM). These two components are cross-linked by short chains of amino acids. In the process of cross-linking of the amino acid chains, two enzymes are involved in this process: D-alanyl carboxypeptidase and transpeptidase. These are sometimes known as penicillin binding proteins (PBPs), and they are the targets for beta-lactam antibiotics. There are two types of PBPs: aPBPs and bPBPs.[24-26,42]. aPBPs are bifunctional and possess both glycosyltransferase (GT) activity for polymerizing the glycan strands and transpeptidase (TP) activity for crosslinking them. bPBPs, on the other hand, are only known to possess TP activity. The primary target of beta-lactams is the TP active site of the synthetic PBPs, which is covalently modified by the drug[27]. The function of beta-lactam antibiotics is to

bind the PBPs that crosslink peptidoglycan and prevent the bacterial cell wall synthesis. Thus, the cell wall becomes weakened and breaks easily due to high intracellular osmotic pressure (figure 3) [29].

Figure 1: The percentage of standard units for each injectable antibiotic prescribed in the United states from 2004 to 2014. (Data from the IMS MDART Quarterly Database on file at Astrazenca) [23].

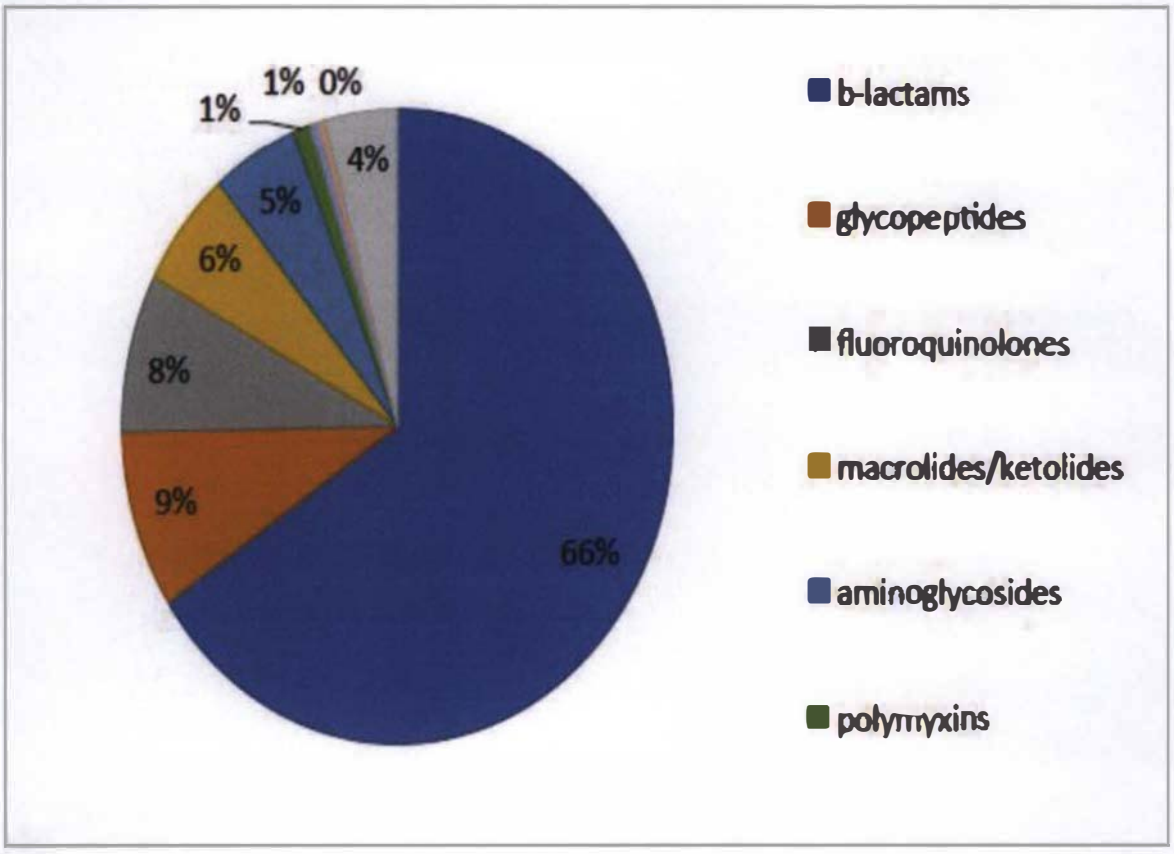
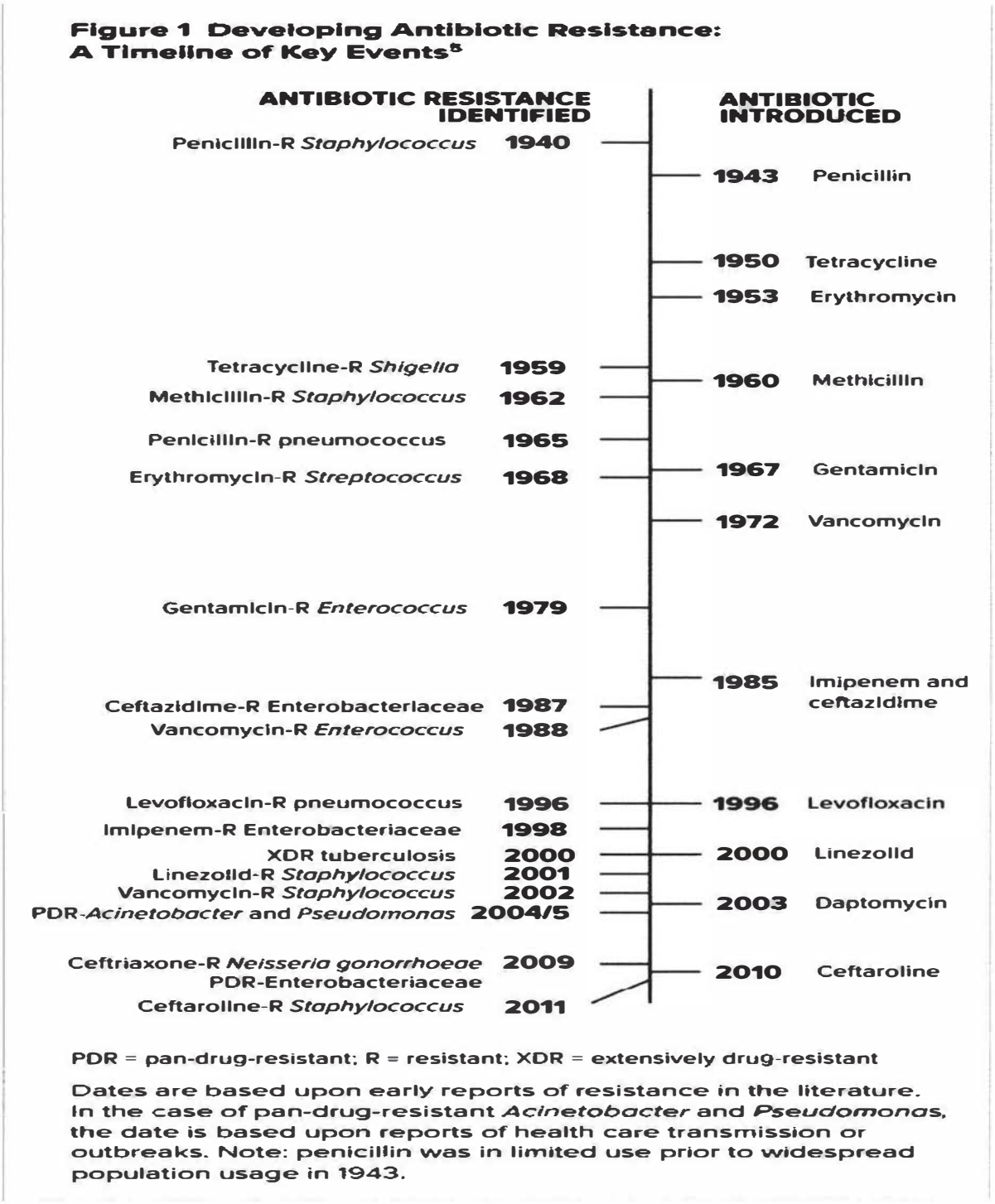


Figure 2: Developing antibiotic resistance; A timeline of key events [23]



1.3 Mechanism of beta-lactam

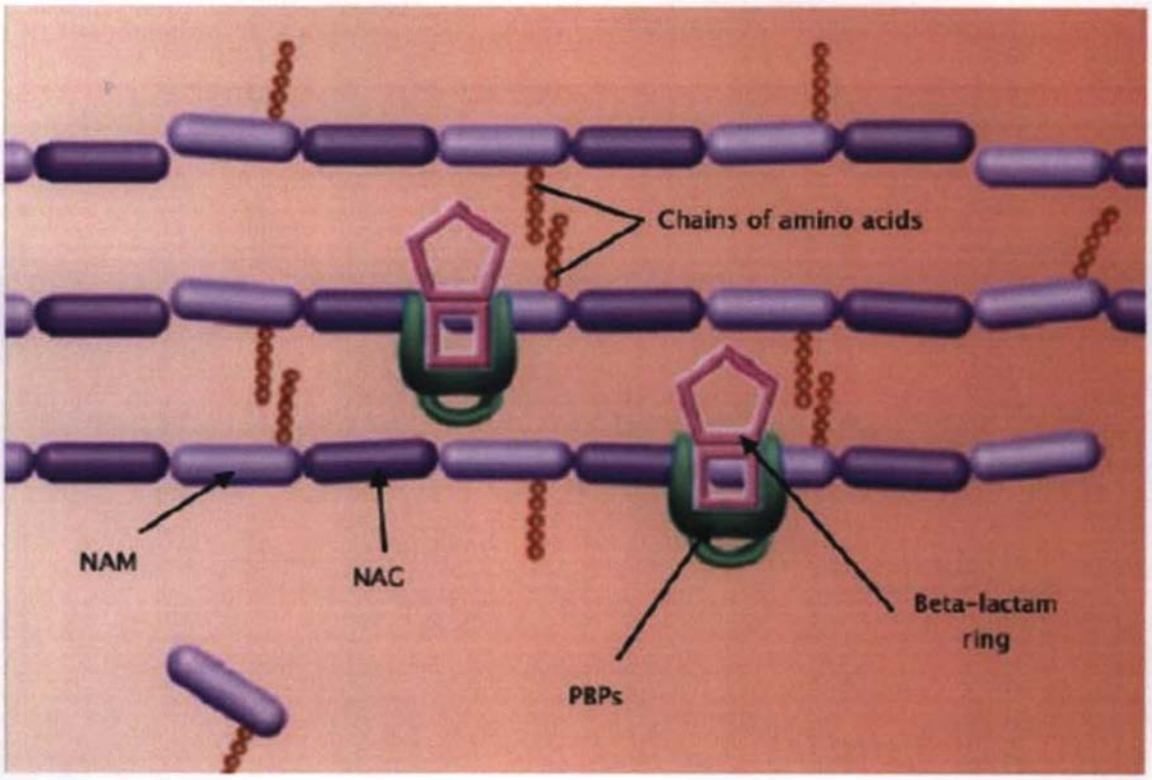


Figure3. Diagrammatic mechanism of beta-lactam drug

Since beta-lactam class drugs are prescribed in high frequency and used routinely as first line antibiotic for infectious diseases, resistance to beta-lactam class drugs poses a significant challenge to human health. The first Beta-lactamase (bla) was recognized in Escherichia coli by E.P. Abraham and E. Chain and named as "penicillinase. Penicillinase was discovered when even the clinical use of penicillin had not been started and the enzyme was not considered to be clinically relevant at the time[27-29,30]. Four years of continuous work lead Kirby to extract these cell-free "penicillin inactivators" from Staphylococcus aureus, which is a significant opportunistic pathogen and he is the first scientist to do it [30].

Beta-lactamase is considered one of the major causes for resistance to large valuable selections of antibacterial agents. It is a group of enzymes formed by various bacterial cells to inactivate beta-lactam antibiotics by destroying the beta-lactam ring structure[figure 4]. There are more than 1,300 beta-lactamases occurring naturally, which make them perhaps one of the largest 15 enzyme families that has been studied. In many clinical treatment regimens beta-lactam class of antibiotics, such as cephalosporins and carbapenems, are used to treat serious infections. The capability of beta-lactamases to inactivate this large array of antibiotics makes them a real threat to public health [31-32].

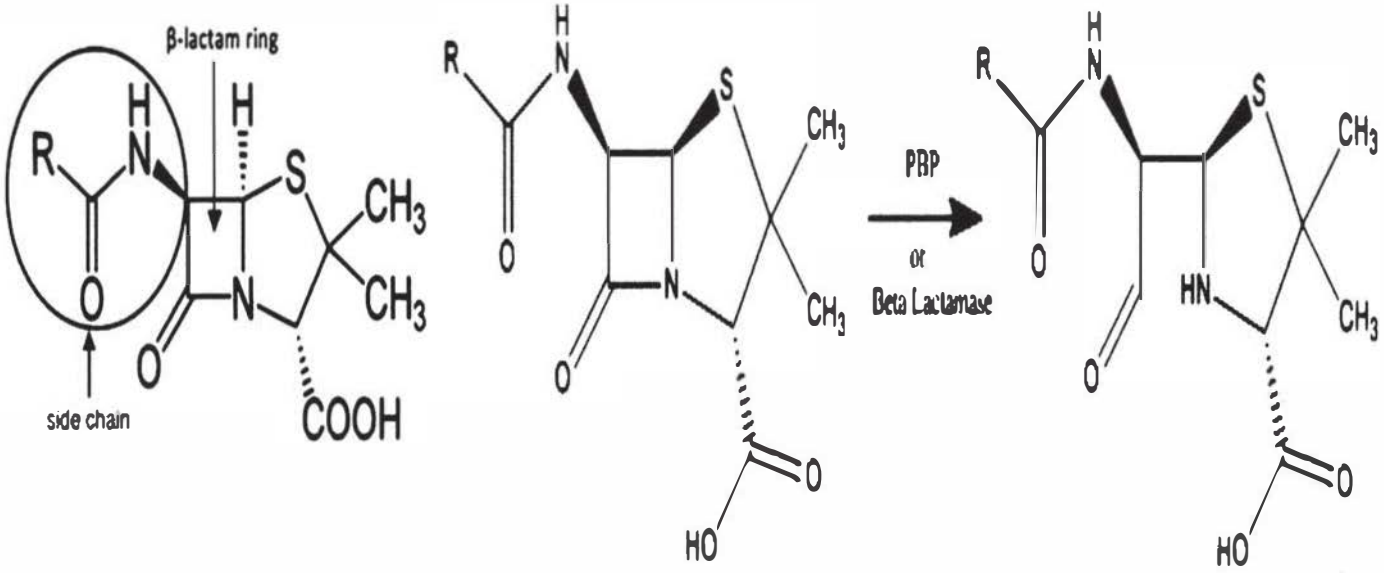


Figure 4: Cleavage of beta-lactamase

There are many mechanisms that bacteria became resistant to beta-lactam, among several others four primary mechanisms occurs most frequently[32]. The very first mechanism is bacteria can produce lactamase enzymes itself. It is very common and significant mechanism of resistance especially in Gram-negative bacteria. Second, by lowering the affinity for beta lactam-antibiotics in which changes can occur in the active site of PBPs. Third, bacteria can decrease the expression of outer membrane proteins (OMPs). Finally, the multidrug resistant occur when efflux pumps can prevent beta-lactam antibiotics to enter the cell or throw them out of the cell [34].

Specifically, in wastewater, there are mainly three lateral gene transfer mechanisms in spreading the beta-lactam antibiotic resistant genes. (I) Conjugation, (II) transformative uptake of chromosomal DNA fragments or plasmids; (III) Transduction mediated by phages [Figure 5]. Once the gene is received and combined, the bacterium can produce beta lactamase and becomes resistant to beta-lactam drugs. Furthermore, once after the treatment the wastewater sample is released back into the environment, if the different resistant variants can persist throughout the treatment, it will then travel with water to other hosts, where different plants and animals are exposed with it and might occur resistant gene directly and indirectly [35].

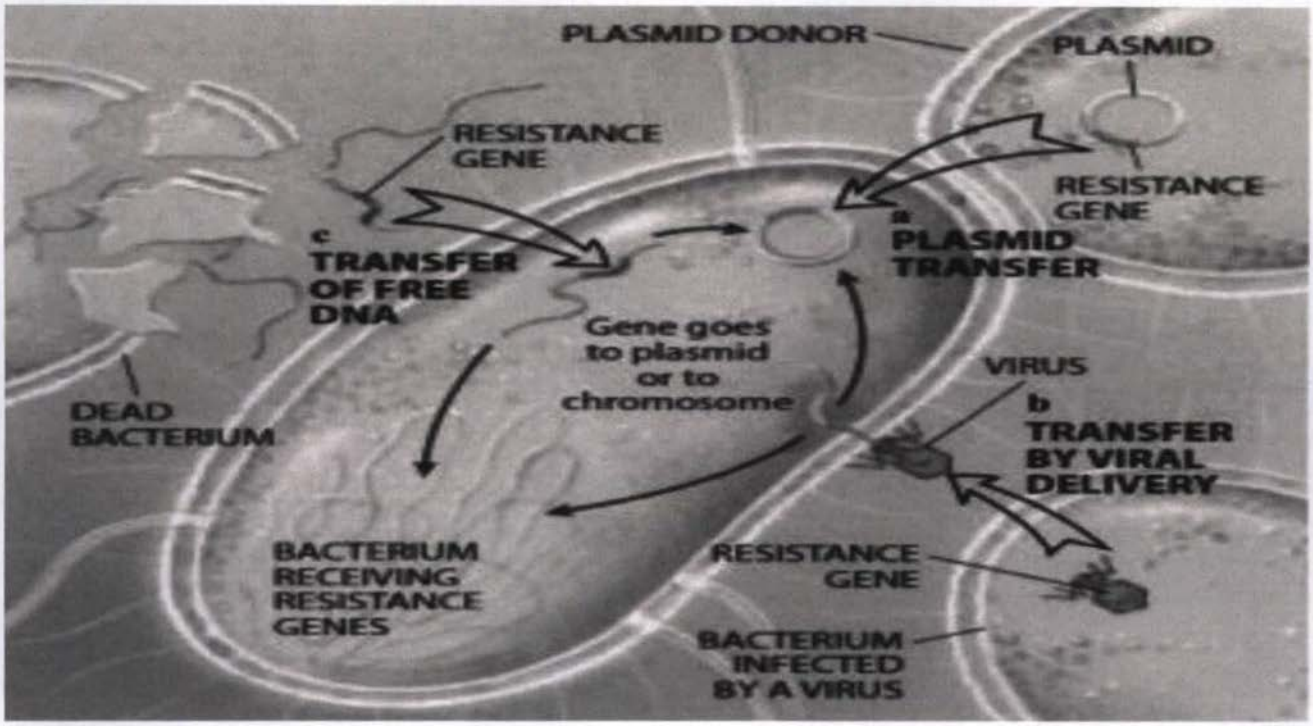


Figure 5: Three main mechanism of transfer of resistant gene[23]

1.4 Classification of beta-lactamase

In 1991, Bush and Medeiros classified the beta-lactamases which is the most common method of classification. Bush's classification is based on the molecular structure of the enzymes in the earlier system . Another scientists Ambler, classified beta-lactamases based on conserved and distinguishing amino acid motifs. This classification is famous and widely used classification introduced in 1991. Both of their classification areß being shown in the Table .

Table 1: Classification of lactamase based on Amber and Bush [36-37]

Amber class	Bush group	Characteristics of beta lactamase	Number of enzymes
A	2a	Staphylococcal and enterococcal penicillinases	23
	2b	Broad spectrum betalactamases including TEM-1 and SHV-1, mainly occurring in gram-negatives	16
	2be	Extended spectrum betalactamases (ESBL)	200
	2br	Inhibitor-resistant TEM (IRT) betalactamases	24
	2c	Carbenicillin-hydrolysing enzymes	19
	2d	Cloxacillin (oxacillin) hydrolysing enzymes	31
	2e	Cephalosporinases inhibited by clavulanic acid	20
	2f	Carbapenem-hydrolysing enzyme inhibited by clavulanic acid	4
B	3	Metallo-enzymes that hydrolyse carbapenems and other betalactams except monobactams. Not inhibited by clavulanic acid	24
C	1	Often chromosomal enzymes in gram-negatives but some are Plasmid-coded. Not inhibited by clavulanic acid.	51

D	4	Miscellaneous enzymes that do not fit into other groups	9

Based on the homology and clinical importance, beta-lactamases can also be classified into different families[Table 2]. Major families of β-lactamases of clinical importance include: *KPC*, *TEM*, *SHV*, *AMPC*, *CTX-M*, *PER*, *VEB*, *GES*, and *CMY*[34-37]. *SHV* family is thought to be primarily derived from Klebsiella spp. and TEM was firstly found in E. coli isolates. These gene families can now be found in multiple enterobacteria species creating a significant threat to treatment [36-28]. The *bla*TEM gene is also one of the most frequently detected plasmid-borne antimicrobial resistance genes, which confers resistance to penicillin's and extended-spectrum cephalosporins [39].

Table 2: Major families of beta lactamase based on clinical importance

Enzyme Families	Functional group	No of enzymes [b,c]	Representative enzymes
CMY	1, 1e	50	CMY-1 to CMY-50
TEM	2b, 2be, 2br, 2ber 2b 2be 2br 2ber	172 12 79 36	 TEM-1, TEM-2, TEM-13 TEM-3, TEM-10, TEM-26 TEM-30 (IRT-2), TEM-31 (IRT-1), TEM-163 TEM-50 (CMT-1), TEM-158 (CMT-9)
SHV	2b, 2be, 2ber 2b 2be 2ber	127 30 37 5	 SHV-1, SHV-11, SHV-89 SHV-2, SHV-3, SHV-115 SHV-10, SHV-72
CTX-M	2be	90	SHV-1, SHV-11, SHV-89
PER	2be	5	PER-1 to PER-5
VEB	2be	7	VEB-1 to VEB-7
GES	2f	15 d	GES-2 to GES-7 (IBC-1) to GES-15
KPC	2f	9	KPC-2 to KPC-10
SME	2f	3	SME-1, SME-2, SME-3
OXA	2d, 2de, 2df 2d 2de 2df	158 5 9 48[e]	 OXA-1, OXA-2, OXA-10 OXA-11, OXA-14, OXA-15 OXA-23 (ARI-1), OXA-51, OXA-58
IMP	3a	26	IMP-1 to IMP-26

VIP	3a	23	VIM-1 to VIM-23
IND	3a	8	IND-1, IND-2, IND-2a, IND-3 to IND-7

[a] Enzyme families based on primary amino acid structures (G. Jacoby and K. Bush, http://www.lahey.org/Studies/).

[b]Compiled through December 2009.

[c]The sum of the subgroups in each family.

[d]GES-1, unlike other members of the GES family, interaction with imepenem

[e]Nine clusters of OXA carbapenem

2. Objective of the Study

This study collected samples from Charleston wastewater treatment plant and nearby upstream and downstream flowing water. In reference to 2010 census the population of the city is 21,838. The aim of the study was to examine the four of the beta-lactamase loci in wastewater and stream sources using PCR with appropriate positive and negative controls.

3. Methods and materials

3.1 Sample collection

Figure 6: Sample collection site, source; google map

I II III IV

Figure 7. Different sampling point:(I) influx; (II); (III) influx bottle and (IV) efflux bottle

Table 3. Sample collection site, wastewater treatment plant and Cassell Creek

Collection Site	Charleston Waste water treatment plant (CWWTP)												Untreated Cassell Creek			
Sample's No	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Sample	Sample	Sample	Sample
Date	02/23/2018		02/28/2018		03/05/2018		3/22/2018		04/05/2018		04/12/2018		04/17/2017		04/12/2018	
Site	Influx	Efflux	Influx	Efflux	Influx	Efflux	Influx	Second	Influx	Efflux	Influx	Efflux	Upstream	Downstream	Upstream	Downstream
Volume	1000ml	2000ml	1000ml	2000ml	1000ml	2000ml	1000ml	2000ml	1000ml	2000ml	1000ml	2000ml	10000ml	1000 – ml	1000 ml	1000 ml
Filtration	No	100ml	No	100ml	No	100ml	No	No	No	100ml	No	100ml	500ml	500ml	500ml	500ml
Centrifugation	250ml	No	250ml	No	250ml	No	250ml	No	250ml	No	250ml	No	500ml	500ml	500ml	500ml

3.2 Procedures

3.2.1. Filtration

Efflux sample, upstream and downstream sample was filtered. Filtration was performed using sterile 47-mm diameter nitrocellulose filters (NALGENE® Nalge Nunc International, USA) with pore size of 0.22 µm. 100mL of sample was filtered at once and collected in single tube with sterile water by using the sterile spatula with scraping. In the second phase of sample collection filter disc were cut into pieces and processed for further examinations.

3.2.2 Centrifugation

Once the sample was were collected, it was processed immediately. Influx sample was centrifuge 250mL each in 4 plastic bottles at 4472xg for 15 min (Sorvall RC-5B Superspeed Centrifuge). After centrifugation, supernatant was removed and mixed in one bottle then distributed in tubes (1.5mL) which were then again centrifuged for 17,000xg for 20 minutes. After that, supernatant liquid was discarded, and solids were combined to reduce the total number of the tubes for the next procedure.

3.2.3 Bead beating and E.Coli Controls

In sample year 2018 four rounds the sample collected was processed with the bead beating tubes provided with Fast DNAR spin kit for soil (MP Biomedicals, USA) in mini-bead beater-16 (BioSpace Products, USA) but no difference was observed in the quality of the DNA. So, after that bead beating was done for five minutes for each sample and *E. coli* controls were kept. Bead beating was done as suggested by manufacture's instruction fast DNA™ spin kit for soil (MP Biomedicals, USA).

Table 4. Time Variation in bead beating

Samples	Tubes Labelled	Time variation each tube (Minute)
Sample 1 Influx	I1	5
	I2	9
	I3	12
Sample 2 Efflux	E1	5
	E2	9
	E3	12
Sample 3 Influx	I1a	5
	I2a	10
	I3a	15
Sample 4 Efflux	E1a	5
	E2a	10

3.2.4 Genomic DNA extraction

Following bead-beating, all samples were processed following manufacturers' protocols according to each respective extraction kit. The total volume of eluted DNA was 100 µL from each single bead beating tube. All the collected sample in sample year 2018 were further cleaned up using GeneClean II kit (MP Biomedicals, USA).

3.2.5 Controls

Purified E. coli strain ATCC 25922 DNA was used as a positive control for all 16s rDNA PCR reactions. Positive controls for *bla* variants were obtained from BEI Resources (Table 9).

3.2.6 Quantification

All the extracted DNA from the samples were quantified by using Epoch™ 2 Microplate Spectrophotometer (BioTek, USA). 2 µL Extracted DNA samples were taken for all quantification tests. Gen5 software was used for this procedure.

3.2.7 Polymerase chain reaction (PCR)

BioRad MyCycler PCR (Hercules, USA) and Biometra T-Gradient Thermal Cycler PCR (Gottingen, Germany) were used in all the PCR reactions. Bacterial DNA were first detected by universal 16s rDNA fragments and different primers set were used to amplify the selected genes. The primers for the 16S rDNA were 47F and 0691R primers (Table 5). Primers for *bla* loci are listed in Table 6. All reactions were carried out using Taq 2X Master Mix (New England Biolab, USA) following the manufacturer's protocols. Each primer was added at 10 pmol per reaction. PCR cycles and conditions are shown in Tables 7 & 8.

Table 5. 16S Universal primers used in this study and sequences.

Primer	Sequence (5'-3')	Reference
47F C GAGTTTGATCCTGGCTCAG	47F C GAGTTTGATCCTGGCTCAG	[40]
0691R GCATTACARGATTTCAC	0691R GCATTACARGATTTCAC	[40]

Table 6. *bl*a primers used in this study sequences.

Primer	Sequence (5'-3')	Reference
*bla*KPC	For: ATGTCACTGTATCGCCGTC Rev: TTACTGCCCGTTAACGCC	[40]
*bla*SHV	For: ATTTGTCGCTTCTTTACTCGC Rev: TTTATGGCGTTACCTTTGACC	[40]
*bla*TEM	For: AGACGTCAGGTGGCACTTTTCGG Rev: CAAGGGGTCTGACGCTCA	[40]
*bla*KPC	For: ATCAAAACTGGCAGCCG Rev: GAGCCCGTTTTATGGACCCA	[40]

Table 7. PCR time and conditions for *16s rDNA* amplifications

Primer sets	Amplifications conditions					
	Initial Denaturation (^{0} c)/Time	Melting (^{0} c)/Time	Annealing (^{0} c)/Time	Extension (^{0} c)/ Time	Final extension (^{0} c)/Time	Hold (^{0} c)
47F 1492 R(Long)	96^0 / 5 mins	96^0 / 30 sec x 35 cycle	49^0 / 20 sec x 35 cycle	72^0 / 1 min 30 sec x 35 cycle	72^0 / 4 mins	4^0
47F 0691R(short)	96^0 / 5 mins	96^0 / 30 sec x 35 cycle	48^0 / 20 sec x 35 cycle	72^0 / 45 sec x 35 cycle	72^0 / 2 min 15 sec	4^0

Table 8. PCR time and conditions for *bla* amplifications

Primers sets	Amplifications conditions					
	Initial Denaturation (^{0} c)/Time	Melting (^{0} c)/Time	Annealing (^{0} c)/Time	Extension (^{0} c)/Time	Final extension (^{0} c)/Time	Hold (^{0} c)
blaKPC blaTEM	94^0 / 1 min	96^0 / 30 sec x 35 cycle	59.5^0 / 30 sec x 35 cycle	72^0 / 60 sec 2 x 35 cycle	72^0 / 3 mins	4^0
blaSHV blaAMPC[a]	94^0 / 1 min	96^0 / 30 sec x 35 cycle	56^0 / 30 sec x 35 cycle	72^0 / 30 sec 45 sec x 35 cycle	72^0 / 3 mins 15 sec	4^0

3.2.8 Agarose gel electrophoresis

Agarose gels (0.8% or 1.4% (w/v)) were prepared in 1x TBE buffer supplemented with GelRed. Electrophoresis was conducted at 95-110 mV for 1-3 hours. Results were viewed with UV illumination, photographed using a Gel Doc XR+ system, then analyzed by Image Lab 3.0 software (BioRad Laboratories, Inc., Hercules, CA, USA).

Table 9. Positive control strain for *bla* variants

Strain	Description	Reference
NR-16464	Klebsiella pneumoniae isolate 1	[40]
NR-16465	Klebsiella pneumoniae isolate 2	[40]
NR-16466	Klebsiella pneumoniae isolate 3	[40]
NR-16470	Klebsiella pneumoniae isolate 7	[40]
NR-16471	Klebsiella pneumoniae isolate 8	[40]

4. Results

4.1 DNA extraction

WWTP water samples, upstream and downstream water samples were collected and processed as described in Methods and Material section. After the sample collection DNA was extracted from fast DNA spinkit for Soil miniprep from MP Biomedicals. This is continued research from previous year so, previously we had used different kit to extract the DNA and figured out MP biomedical kit was good for this kind of environmental sample for extraction. In the process time duration were varied in each sample of bead beating to check the quality of DNA[Table 4]. Stock E. coli solutions were added, to control for the ability of the kits to obtain bacterial DNA, and the solutions were added to select samples prior to processing.

After the purification with the MP Fast DNA Spin kit, samples were further purified using GeneClean II system from MP Biomedicals to remove putative chemical inhibitors for PCR reactions. DNA samples were then quantified as described (Table 10). The 260/280 ratio was also obtained to assess the purity of the DNA samples. In general, influx samples yielded higher amounts of total DNA. The duration of bead beating did not make significant changes in the quantity of DNA retrieved, nor did it make a significant change in the purity ratio.

4.2. PCR Results

4.2.1 Bacterial 16s rDNA detection

In wastewater besides bacteria there are many other organisms. The presence of total DNA of good quality is not enough indication that they are suitable for PCR based assay. Therefore, as a control to show that DNA samples we extracted contain bacterial chromosomal DNA, the 16s rDNA universal primers were used. Short pair of 16S fragment were used as control reactions for the presence of bacterial samples (Figures 10). Successful amplification of the 16S pair fragments indicates that there are detectable levels of bacterial chromosomes in the extracted total DNA of a preparation.

In the beginning certain extraction methods PCR amplification were failed in 16Sr DNA so we used the additions of positive *E. Coli* to the environmental DNA samples (Figure 8). The results showed that the same amount and type of DNA that can produce successful 16S amplification on its own did not lead to detectable PCR products when added to the environmental DNA samples extracted using either the e ZR-Duet DNA/RNA miniprep or the Fecal DNA Sample miniprep kit

but after using Fast DNA spin kit for soil(MP Biomedicals, USA) we are able to successfully amplify the 16s rDNA.

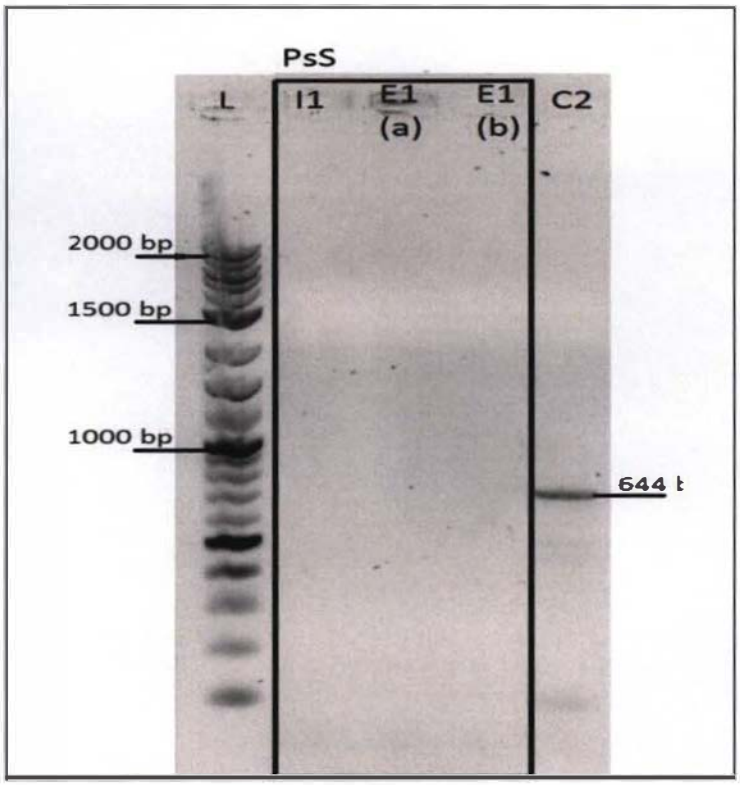


Figure 8. Detection of the short pair of 16S rDNA gene coding for the beta-lactamase in DNA isolated form different waste water sample. E1 and E2 are efflux sample and C2 is the *E.Coli* positive Control.

Table 10. DNA quantification of waste water and untreated upstream

Sample	Labelled tube	DNA concentration(ng/µl)	260/280 ratio
Klebsiella Pneumoniae control			
Sample 1 : Influx	I1a	184.83	1.54
	I1b	95.92	1.81
	I1c	88.99	1.76
Sample 2: Efflux	E1a	140.23	1.93
	E1b	110.57	1.93
	E1c	97.31	1.88
	E1d	210.32	1.77
Sample 3: Influx	I2a	92.53	1.40
	I2b	65.50	1.81
Sample 4:Efflux	E2a	130.63	1.54
	E2b	92.18	1.33
Sample5: Upstream	U1a	21.18	2.60
	U1b	18.42	2.11
Sample6: Downstream	D2a	32.49	3.14
	D2b	42.32	2.28
	D2c	26.61	2.21

Based on the 260/280 ratio so we use 100 ng/µl as standard to calculate our genomic DNA and based on these calculations we have set up our PCR reaction. In addition to the 260/280 ratio, which reveals the amount of protein carry over in the DNA preparation, the quality of the total DNA samples was also assessed using agarose gel electrophoresis.

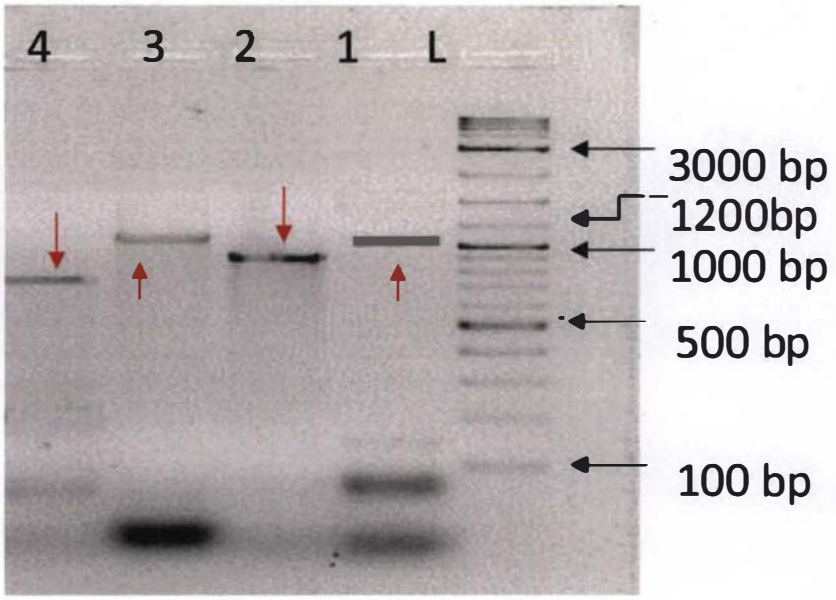


Figure 9. Detection of 4 different *bla* variants control, Lane L is the DNA size marker, lane 1 is the *bla*SHV gene with 1100 bp, lane 2 is *bla*KPC gene with lane size 890bp, lane 3 is *bla*TEM gene with lane 1150 bp and 4 is *bla*AmpC gene with lane 800bp size. Position of each *bla* gene is indicated arrow.

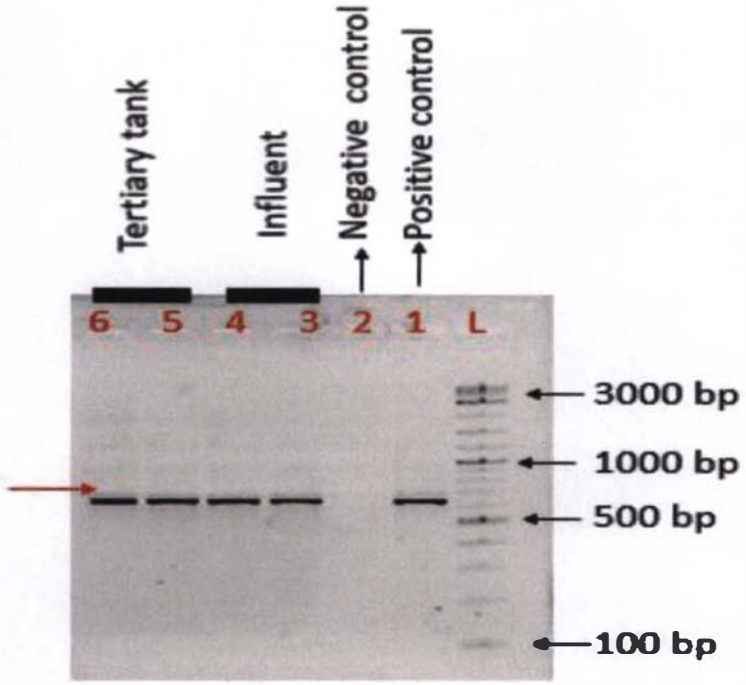


Figure 10. Detection of the *16s rDNA* gene coding for the beta-lactamase in DNA isolated from different waste water samples. Lane L: DNA size marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3 & 4: influent source(644bp); Lane 5 & 6: tertiary tank source. Position of the *16s rDNA* gene is indicated by red arrow.

4.2.2 *bla* variants detection

bla variants were detected and protocol was carried out as described in Methods and Material, with positive control DNA obtained from the BEI Resource Center. The four variants of bla were each assayed in separate PCR reactions using

different samples from collection sites. The sample was collected from influx, efflux, tertiary tank, downstream water and upstream water.

All four variants *bla*KPC, *bla*TEM, *bla*SHV and *bla*TEM were detected in influx and tertiary tank samples, whereas none of the efflux or stream water samples showed positive outcomes (Figures 11-14). In the sample year 2017 For variant *bla*TEM, two of the four wastewater samples showed a positive amplification while none of the influx or stream water samples did . The PCR results of 16s rDNA and *bla* variants from 2017-2018 is summarized in Table11a and 11b.

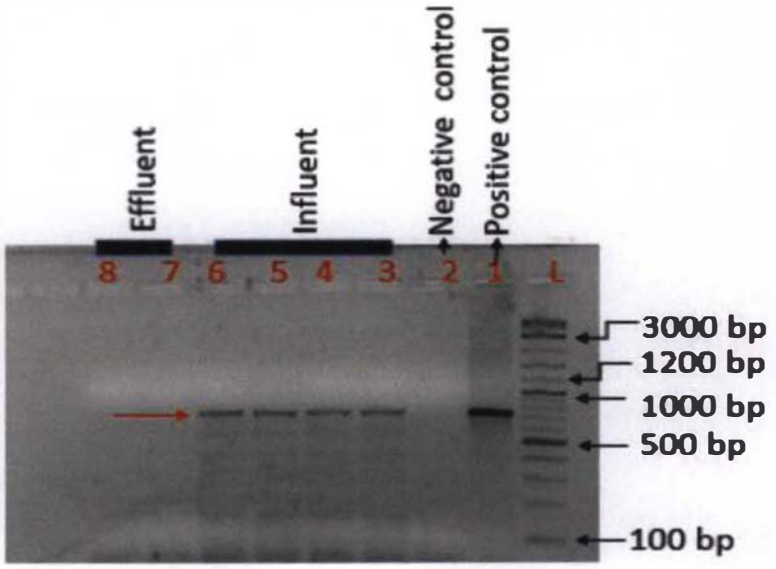


Figure 11. Detection of the *bla*AmpC gene coding for the beta-lactamase in DNA isolated from different waste water samples. Lane L: DNA size marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3-6: influent source(780bp); Lane 7 & 8: effluent source. Position of the blaAmpC is indicated by arrow.

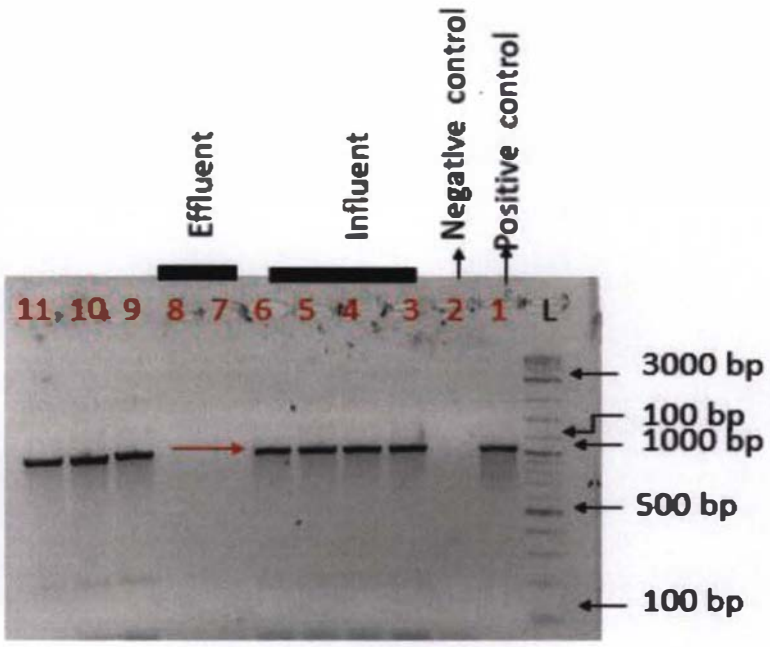


Figure 12. Detection of the *bla*SHV gene coding for the beta-lactamase in DNA isolated from different waste water samples. Lane L: DNA size marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3-6: influent source(1100bp); Lane 7 & 8: effluent source. Lane 9-11: Influent source; Position of the blaSHV is indicated by arrow.

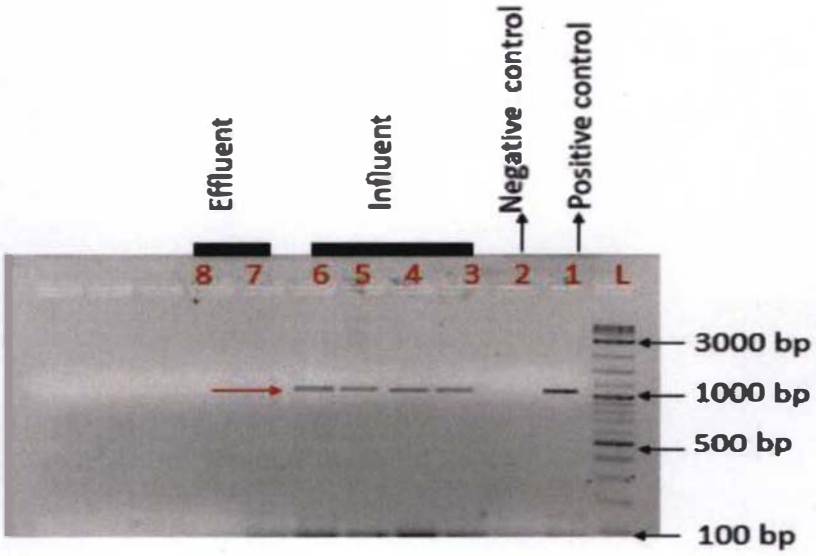


Figure 13. Detection of the blaTEM gene coding for the beta-lactamase in DNA isolated from different waste water samples. Lane L: DNA size marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3-6: influent source(1150bp); Lane 7 & 8: effluent source. Position of the blaKTEM is indicated by arrow.

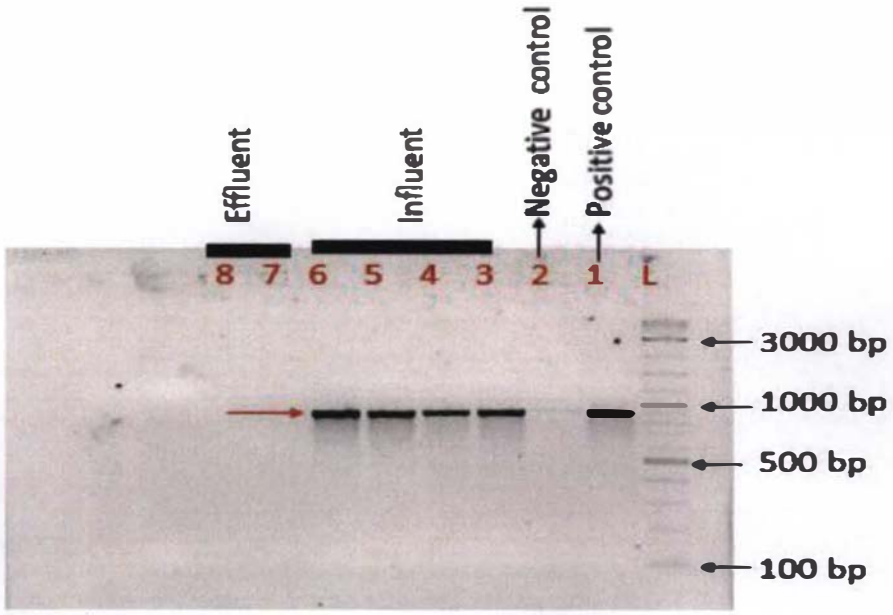


Figure 14. Detection of the *bla*KPC gene coding for the beta-lactamase in DNA isolated from different waste water samples. Lane L: DNA size marker; Lane 1: Positive control; Lane 2: Negative control; Lane 3-6: influent source(890bp); Lane 7 & 8: effluent source. Position of the blaKPC is indicated by arrow.

Table 11a. PCR summary; Detection of beta lactamase in sample year-2017

Samples and control		bla variants			
	47F 0691R	blaKPC	blaTEM	blaSHV	blaAmpc
E. coli	+	-	-	-	-
Klebsiella Pneumoniae	+	+	+	+	+
Influx	+	+	+	+	+
Efflux	+	-	-	-	-
Downstream	+	-	-	-	-
Upstream	+	-	-	-	-

Table 11b. PCR summary; Detection of beta lactamase in sample year-2018

Samples and control		bla variants			
	47F 0691R	blaKPC	blaTEM	blaSHV	blaAmpc
E. coli	+	-	-	-	-
Klebsiella Pneumoniae	+	+	+	+	+
Influx	+	+	+	+	+
Tertiary tank	+	+	+	+	+
Efflux	+	-	-	-	-

5. Discussion

In the study we used; FastDNA ^{R} spin kit for soil, MP Biomedicals which gives the good concentration of DNA (Table 10) and also amplify the 16s rDNA genes (figure 10). However, in the first phase of sample collection we were not able to extract the concentration of DNA[43]. Later on, the second phase of sample collection we figured it out using the extraction kit from MP Biomedicals using the gene clean kit after the PCR process.

Furthermore, in the first phase of sample collection *E.Coli* DNA was added to the extracted sample as template for PCR, no positive outcomes were detected[43]. This result demonstrated that the two extraction kits allowed PCR inhibitors to be carried through the samples during processing in the first sample year[43].

This result showed that the purification process managed to remove PCR-inhibiting compounds from the samples that the two other kits left behind. For all three DNA extraction kits examined[43], the duration of bead-beating did not seem to make a significant difference in the outcome, so we did 5 min of bead beating for every sample in the second phase of sample processing. In all cases, lengthening the duration did not cause any observable changes in the quality of DNA samples and had no measurable impacts on the success of PCR amplifications.

5.1 Presence of *bla* variants in water samples

Four ARGs *bla*TEM, *bla*KPC, *bla*SHV, *bla*AmpC and the 16S rDNA gene were amplified using PCR assays on samples collected at the different stages of wastewater treatment as well as from fresh water samples. All samples examined showed positive outcomes for the 16S rDNA amplification, thus confirming the presence of detectable levels of bacterial DNA in all samples. Further, PCR control amplifications with the 4 primer pairs specific to the 4 *bla* variants using *Klebsiella* spp. genomic DNA produced PCR amplicons, demonstrating that our PCR primer pairs are capable of amplifying the target genes. For wastewater and fresh water samples, all four *bla* variants were detected in influx, and tertiary tank. However, these were not detected on downstream and upstream water and efflux samples. Since 16S rDNA amplicons were present in all samples, the absence of variant-specific amplification products in our samples suggested that these genetic materials were present at a level that is below detection in those samples. The absence of detectable levels of *bla* variants in the efflux samples showed that

water treatment process successfully reduced the level of these DNA loci to a level that is below PCR detection. The stream water outcomes also demonstrated that these loci are more common in the wastewater influx, and not typically present in untreated stream water.

Although the four loci were no longer detectable at the efflux sample, there is still potential hazard in their presence in the influx and up to tertiary tank sample. Throughout the wastewater sample processing, solid wastes are removed and treated in different ways, such as being used as starting material for anaerobic digester. These resistant loci may persist in those venues. Further examination of the wastewater treatment process will be required to fully monitor the potential health risks. Also, this study focused on one sample taken at one time during the processing. Therefore, the results should not be generalized to reflect the overall status of the quality of the wastewater. Further examination of the process (different sample sites) with routine sampling is necessary to understand the picture fully. There are also other bla genes of importance for human and animal. Future research should focus on these gene and the resistance bacteria. Also, the next step is that after understanding which organisms carry ARGs, and how mobile these genes are, we can make evidence-based conclusions on the risk caused by antibiotic resistance in waste waters and the possible mitigation of those risks. By saying this a continuous monitoring procedure with regular sampling will be required to fully understand the risk of a significant presence of these resistant loci in our wastewater samples.

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